Revista Brasileira de Engenharia Biomédica,
v. 23, n. 2, p. 123-130, abril 2007
© SBEB - Sociedade Brasileira de Engenharia Biomédica
ISSN 1517-3151

Artigo Original
Recebido em 03/01/2007, aceito em 11/04/2007

Instrumentation for measurement of intracellular Ca^{2+} concentration: Effects of beta-adrenergic stimulation on isolated cardiac myocytes

Instrumentação para medição da concentração intracelular de Ca^{2+}: Efeitos da estimulação beta-adrenérgica em miócitos cardíacos isolados

Diogo Coutinho Soriano
Rosana Almada Bassani
José Wilson Magalhães Bassani*
Departamento de Engenharia Biomédica,
FEEC/UNICAMP
Caixa Postal 6040
13084-971 Campinas - SP, Brasil
E-mail: bassani@ceb.unicamp.br

*Corresponding author

Abstract
Ca^{2+} ions play an important role in signaling and regulation of several cell processes. In the myocardium, Ca^{2+} is fundamental for generation and modulation of electrical activity, as well as for contraction initiation. In this article, we report a microfluorimetry/microscopy system that allows simultaneous measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and mechanical activity (cell shortening) in cardiac myocytes. The main advantages of the developed instrument are compactness, simplicity, easy operation and low cost, in comparison to commercially available equipment. The recorded fluorescence signal showed acceptable signal-to-noise ratio, and the expected amplitude range for the Ca^{2+} indicator fluo-3. The instrument was applied to the analysis of the positive inotropic and lusitropic effects of β-adrenergic stimulation on isolated rat ventricular myocytes. Contractions and $[Ca^{2+}]_i$ transients were simultaneously recorded from fluo-3-loaded myocytes electrically stimulated at 0.5 Hz at room temperature, in the absence and presence of the β-adrenoceptor agonist isoproterenol (ISO, 10 nM). ISO significantly increased $[Ca^{2+}]_i$ transient and contraction amplitude, and accelerated $[Ca^{2+}]_i$ decline and mechanical relaxation ($p < 0.05$). The demonstration of the well-known positive inotropic and lusitropic effects of β-adrenergic stimulation by such a low agonist concentration shows that the instrument allows detection of simultaneous changes in $[Ca^{2+}]_i$ and mechanical activity in cardiac myocytes. This instrument may be used for $[Ca^{2+}]_i$ measurement in other cell types, such as neurons, other types of muscle cells, and gland cells.
Keywords: Rat ventricular myocytes, Biomedical instrumentation, Intracellular calcium concentration measurement, Fluo-3, Adrenergic stimulation, Cell shortening, Calcium transients.

Resumo

O íon Ca^{2+} desempenha um importante papel na regulação e sinalização de diversos processos celulares. No músculo cardíaco, Ca^{2+} é fundamental para a geração e modulação da atividade elétrica, bem como para o processo de contração. Este trabalho apresenta um sistema de microfluorimetria e microscopia que permite a medição simultânea da concentração citosólica de Ca^{2+} ($[Ca^{2+}]_i$) e da atividade mecânica (encurtamento celular) em miócitos cardíacos. A instrumentação desenvolvida apresenta a vantagem de ser de pequeno porte, de fácil operação, pouco complexa e de baixo custo, quando comparada com os equipamentos comerciais disponíveis. O sinal de fluorescência registrado apresentou boa relação sinal-ruído e amplitude compatível com a esperada para a emissão do indicador de Ca^{2+} utilizado (fluo-3). O sistema foi aplicado para analisar os efeitos inotrópico e lusitrópico positivos da estimulação β-adrenérgica em miócitos ventriculares isolados de rato. Em células carregadas com fluo-3, transientes de $[Ca^{2+}]_i$ e contrações foram registrados de forma simultânea durante estimulação elétrica à freqüência de 0,5 Hz a 23 ºC, na presença e na ausência de 10 nM do agonista de adrenoceptores β isoproterenol (ISO). ISO causou aumento significativo da amplitude dos transientes de $[Ca^{2+}]_i$ e das contrações, bem como aceleração da queda de $[Ca^{2+}]_i$ e do relaxamento ($p < 0,05$). A demonstração dos conhecidos efeitos inotrópicos e lusitrópicos positivos da estimulação β-adrenérgica com baixa concentração do agonista indica que o sistema apresenta sensibilidade satisfatória para detecção de variações simultâneas em $[Ca^{2+}]_i$ e na atividade mecânica de células cardíacas. A utilização do presente sistema, para medição de $[Ca^{2+}]_i$, não está restrita a este tipo celular, mas pode ser estendida a outros tipos celulares (e.g., neurônios, outros tipos de células musculares, células glandulares).

***Palavras-chave**: Miócitos ventriculares de rato, Instrumentação biomédica, Medição de concentração intracelular de Ca^{2+}, Fluo-3, Estimulação adrenérgica, Encurtamento celular, Transiente de cálcio.*

Introduction

Ca^{2+} ions play a major role in signaling and regulation of cellular processes, such as cell division and proliferation, neurotransmitter release, gene transcription and apoptosis (Berridge *et al*., 2000). In the cardiac muscle, Ca^{2+} is fundamental to the generation of electrical activity at the nodal pacemaker and in shaping the action potential (AP) waveform, and especially to the control of the contraction process (Bers, 2002). Sarcolemmal depolarization during the AP activates Ca^{2+} influx and consequently the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the Ca^{2+} induced Ca^{2+} release mechanism (Fabiato, 1983). Ca^{2+} released from the SR (the main source of Ca^{2+} for contraction in cardiac myocytes – Bers, 2001) leads to increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which is an essential condition for contraction development. When more Ca^{2+} becomes available to the myofilaments, it binds to troponin C, which undergoes conformational changes that result in greater interaction between actin and myosin. This process leads to cross-bridge formation and cycling, and ultimately to contraction development. On the other hand, Ca^{2+} removal from the citosol is required for relaxation (Bers, 2001). Therefore, under physiological conditions, contraction is triggered by excitation, and Ca^{2+} couples these two processes.

The study of Ca^{2+}-dependent processes, such as excitation-contraction coupling, usually requires $[Ca^{2+}]_i$ quantitation, which is commonly achieved with the aid of fluorescent Ca^{2+}-binding dyes (Takahashi *et al*., 1999; Thomas and Delaville, 1991). These dyes, usually derived from divalent cation chelators, are introduced in the cytosol (either by passive diffusion of membrane-permeant forms or via an impaling micropipette, in the case of charged forms) and have their spectral properties altered upon binding to Ca^{2+}. Under adequate optical excitation, they emit light in a $[Ca^{2+}]_i$ dependent way. The emitted light may be measured and converted to $[Ca^{2+}]_i$ using specific calibration parameters (Gomes *et al*., 1998), which allows monitoring the amplitude and timecourse of $[Ca^{2+}]_i$ variations under a variety of experimental conditions (Bassani *et al*., 1992, 1993, 1994; Bassani and Bassani, 2002; Carvalho *et al*., 2006).

The present work describes a microfluorimetry system that integrates a microscopy device based on a system previously developed in this laboratory (Ricardo *et al*., 2006), which allows simultaneous measurement of mechanical activity and $[Ca^{2+}]_i$ (using the fluorescent dye fluo-3) in isolated ventricular cardiomyocytes. The developed instrumentation was applied to analyze

the well-known positive inotropic (i.e., enhancement of contraction amplitude) and lusitropic (i.e., acceleration of relaxation) effects of the β-adrenoceptor agonist isoproterenol (ISO), and compare the mechanical and $[Ca^{2+}]_i$ responses to the agonist.

Methods

Instrumentation for simultaneous $[Ca^{2+}]_i$ and cell shortening measurement

The complete microfluorimetry/microscopy system used in this work was developed at the Center for Biomedical Engineering (CEB), UNICAMP, and is composed of 3 main parts: dye excitation optics, emission collection optics and image projection optics. Fluo-3 is excited by blue light (450-500 nm) and emits fluorescence in a $[Ca^{2+}]_i$-dependent way at the wavelength range of 515-560 nm (Kao *et al.*, 1989). Figure 1 illustrates the components of the instrument.

a) *Excitation Optics*: A blue high-intensity LED (light-emitting diode, OT16-5100-BL, Opto Technology, Wheeling, USA) was used as the light source for fluo-3 excitation. The excitation beam could be blocked between measurements by a manual shutter, to minimize dye photobleaching (Song *et al.*, 1995). The optical condenser, formed by two identical plane-convex lenses (35 mm focal distance, 25 mm diameter; Optovac, Osasco, Brazil), was used for maximizing the collected light. A narrow band optical filter (Chroma Technology Corp., Rockingham, USA) was used to select the excitation bandwidth (460-500 nm). A biconvex lens converges the excitation light beam onto the objective. The diameter of the excitation light beam is limited by a diaphragm (Carl Zeiss, Oberkochen, Germany) that reduces its interference on the photodetector. A dichroic mirror (Chroma Technology Corp., Rockingham, USA) placed at 45° splits the incident light beam and reflects blue light (wavelengths below 500 nm) toward the biological sample, whereas the emitted light (515-560 nm) is transmitted through the mirror. Finally, a 40× magnification objective (Olympus, Melville, USA) works as a condenser of radiant power to the biological sample.

b) *Emission Collection Optics*: The objective collects the emitted light and projects it onto the photodetector, which is a photomultiplier tube (PMT) R4220 (Hamamatsu Photonics, Shizuoka, Japan). The emission wavelength band is restricted not only by the dichroic mirror and the red reflecting mirror (Edmund Optics, Barrington, USA), but also by a narrow band interference filter (515-560 nm, Chroma Technology Corp., Rockingham, USA) placed in front of the PMT.

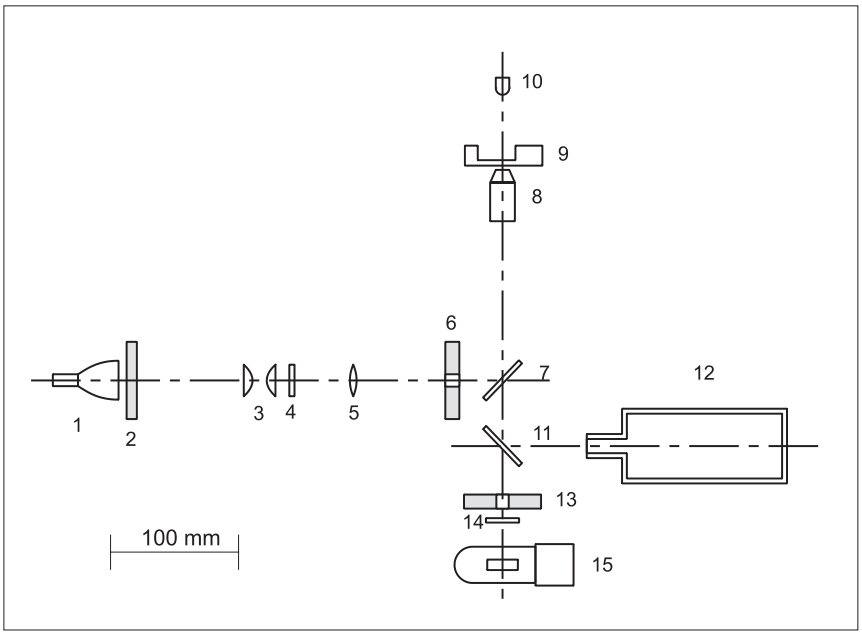

Figure 1. Components of the integrated microfluorimetry/microscopy system for $[Ca^{2+}]_i$ and cell shortening measurement: high-intensity blue LED (1); shutter (2); condenser (plano-convex lenses) (3); excitation narrow band optical filter (4); biconvex lens (5); diaphragm (6); dichroic mirror (7); objective lens (40×) (8); perfusion chamber (9); red LED (10); red reflector mirror (11); CCD camera (12); optical barrier with rectangular slit (13); emission narrow band optical filter (14); photomultiplier tube (15). The scale allows estimation of the actual size and distance among the components of the system.

The aperture of a rectangular slit positioned in front of the PMT allows restriction of the region of the cell from which emission is acquired. The PMT voltage output, which is proportional to the intensity of the detected light signal, was acquired to a computer via an acquisition board PCI 6229 (National Instruments, Austin, USA). Labview software (7.1, National Instruments, Austin, USA) was used for signal recording, displaying and processing.

c) *Image Projection Optics*: A red LED is used to illuminate the biological sample. The image is collected and magnified by the objective, and projected by the red mirror reflector to a CCD (charge coupled device) camera (ICD-31, Ikegami Tshusrink Co., Tokyo, Japan). For more details on the microscopy system, see Ricardo *et al*. (2006).

The acquired video signal was fed to a videocassette recorder (VCR), and the VCR video output fed a video edge detector (VED, developed at CEB, UNICAMP, Patent #PI0300834-7), of which the voltage output is proportional to the displacement of a dot positioned at the cell edge. Both the cell image and the VED tracking signal were displayed on a video monitor. The VED output signal was acquired by the same acquisition board used for PMT signal acquisition. Calibration of the VED output as a function of edge displacement was performed with the aid of a micrometric graticule. The visual field of the microscopy system is 60 × 80 μm in size, and the total magnification of the system is 3,000 times.

The voltage signals from the PMT and the VED were sampled at 100 Hz and filtered at 25 Hz by a 8 pole low-pass Butterworth filter.

Myocyte Preparation and Experimental Protocol

Ventricular myocytes were isolated enzymatically from adult male Wistar rats by coronary perfusion with collagenase type I (0.5 mg/ml, Worthington Biochem. Corp., Lakewood, USA), followed by mechanical dissociation, as described by Bassani and Bassani (2002). The protocol was approved by the institutional Ethics Committee for Animal Experimentation (Proc. #952-1).

Cells were plated onto a perfusion chamber (CEB/UNICAMP, Patent #PI0302.403.2) especially adjusted to fit on the stage of the microscopy-microfluorimetry system. Myocytes were perfused with modified Tyrode's solution (NT), which had the following composition (mM): 140 NaCl; 6 KCl; 1.5 $MgCl_2$; 5 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES); 11.1 glucose; 1 $CaCl_2$; pH 7.4 at 23 °C adjusted with NaOH. Myocytes were electrically stimulated at 0.5 Hz with bipolar voltage pulses (amplitude 1.2× threshold, 5 ms duration) delivered by an electrical stimulator developed at CEB/UNICAMP. Cells were loaded with fluo-3 (acetoxymethyl ester, 1 μM, Molecular Probes, Eugene, EUA) in the dark and in absence of electrical stimulation for 20 min. After loading, cells were perfused for 20 min with NT to allow fluo-3 washout and deesterification.

Fluorescence and cell shortening were simultaneously recorded before and after 3 min perfusion with NT containing the 10 nM (-)isoproterenol (ISO, Sigma Chem. Co., St. Louis, USA).

Measurements and Statistical Analysis

Fluorescence was converted to $[Ca^{2+}]_i$ by the expression (Cheng *et al*., 1993):

$$[\mathrm{Ca}]_i = \frac{\frac{F - F_{back}}{F_0 - F_{back}} \cdot K_{d(\mathrm{Ca})}}{\frac{K_{d(\mathrm{Ca})}}{[\mathrm{Ca}]_d} - \frac{F - F_{back}}{F_0 - F_{back}} + 1} \quad (1)$$

(1)

where F represents the measured emission; F_0 is the diastolic emission; F_{back} is the background emission (i.e., from an empty microscopic field); $K_{d(Ca)}$ is the apparent Ca^{2+} dissociation constant of the dye (1.1 μM; Harkins *et al*., 1993) and $[Ca^{2+}]_d$ is the diastolic Ca^{2+} concentration (assumed as 0.23 μM; Bassani and Bassani, 2002).

Contraction amplitude was considered as the peak systolic cell shortening (expressed as a percentage of the resting cell length, RCL, which, by its turn, was estimated from direct measurement on the video monitor and the image amplification factor). Ca^{2+} transient amplitude was the difference between peak systolic and average diastolic $[Ca^{2+}]_i$ values. ISO lusitropic effects were assessed by analysis of the half-time values for cell relengthening (mechanical relaxation) and $[Ca^{2+}]_i$ decline.

Data from 7 cells were expressed as mean ± standard error, and compared by Student's t-test for paired samples. Differences were considered as statistically significant if $p < 0.05$.

Results

Figure 2 shows the integrated microfluorimetry/microscopy system. The developed instrument is compact, when compared to the equipment commercially available. This is a consequence of the low complexity of the system that does not include monochromators, optical fibers or arc lamps, which also resulted in a low-cost instrument.

Figure 3 shows a typical cardiac myocyte as seen in the video monitor. Only the cell portion displayed in the figure was illuminated by the blue light. The image of the cell free edge allowed tracking its movement for measurement of contraction amplitude. The recorded fluorescence signal showed a good signal-to-noise ratio, and the ratio between peak systolic and diastolic fluorescence was similar to that reported in the literature (~2.0 – Shannon *et al*., 2002).

Simultaneously recorded Ca^{2+} transients and contractions evoked by electrical stimulation are shown in Figure 4A. It can be clearly seen that $[Ca^{2+}]_i$

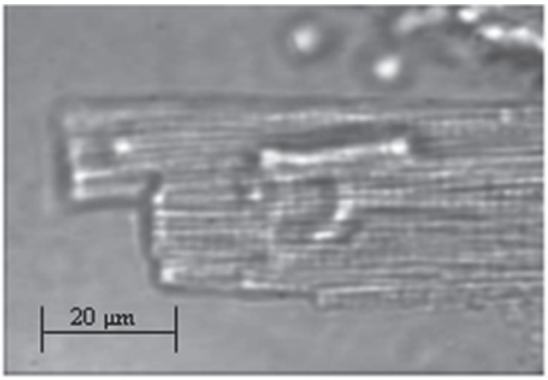

Figure 3. Visual field (60 × 80 µm) of the microfluorimetry/microscopy system, in which a ventricular myocyte is partially displayed. The cell image is intentionally out of focus, to allow better tracking of the edge movement.

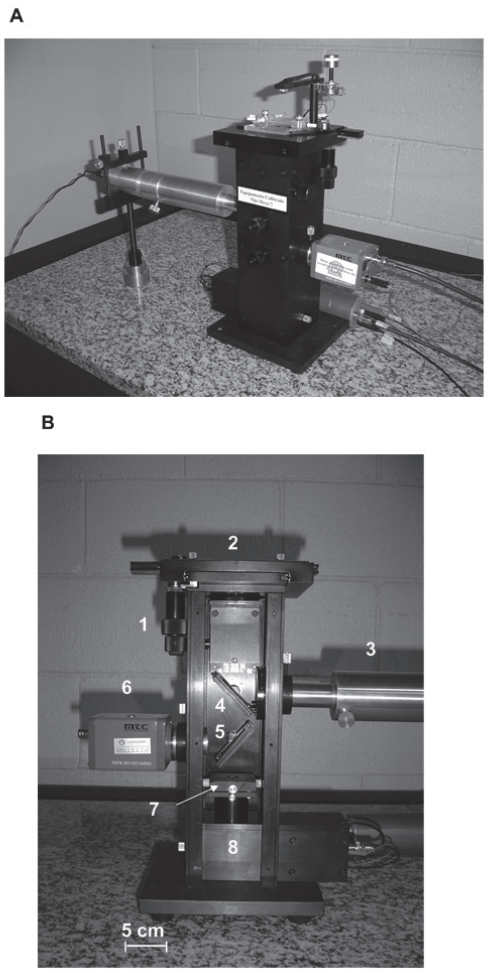

Figure 2. (A) integrated microfluorimetry/microscopy system. (B) the main body of the instrument was open as to reveal: 1) knobs for x-y adjustment of the microscope stage; 2) stage; 3) illuminator containing lenses (condenser and biconvex), excitation narrow band optical filter and light source; 4) inclined dichroic mirror; 5) red reflecting mirror; 6) CCD camera; 7) optical barrier with rectangular slit; 8) photomultiplier tube (inside the box).

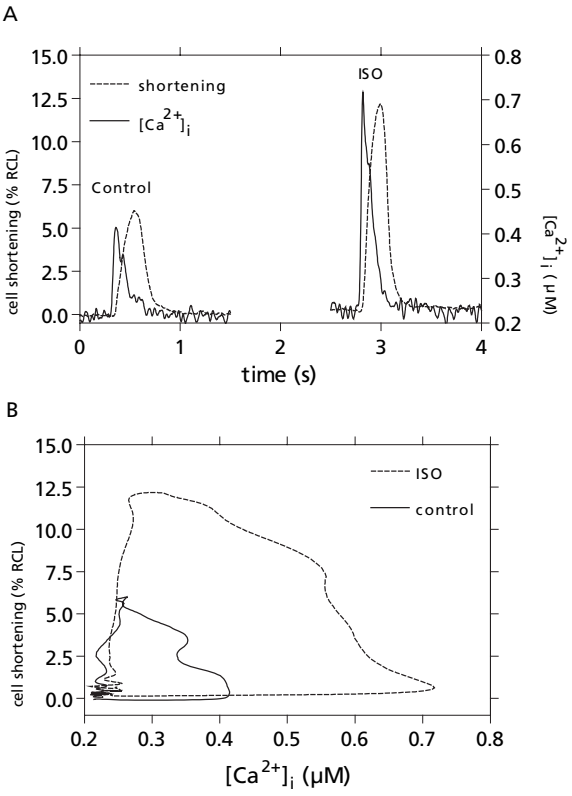

Figure 4. ISO effects on Ca^{2+} transients and contractions of rat ventricular myocytes. (A) $[Ca^{2+}]_i$ and cell shortening (as % of resting cell length, RCL) traces recorded simultaneously from a myocyte in the absence and in the presence of 10 nM isoproterenol (ISO). (B) shortening-$[Ca^{2+}]_i$ loop during an single steady-state twitch at 0.5 Hz under both experimental conditions.

increase preceded development of mechanical activity. Figure 4A also shows the effects produced by ISO, namely increase in the amplitude of $[Ca^{2+}]_i$ transients and contractions (positive inotropic effect), and acceleration of $[Ca^{2+}]_i$ decline and relaxation (positive lusitropic effect). Figure 4B depicts the explicit relation between $[Ca^{2+}]_i$ and cell shortening, which could be determined because of the possibility of simultaneous measurement of both signals. ISO expanded the shortening-$[Ca^{2+}]_i$ loop both rightward and upward (i.e., enhancing the amplitude of $[Ca^{2+}]_i$ and cell length variation, respectively) in an approximately proportional way, but did not cause marked shift or change in the shape of the loop.

In a total of 7 cells, 10 nM ISO increased the amplitude of $[Ca^{2+}]_i$ transients and contractions by 90%, and decreased the $t_{1/2}$ values for $[Ca^{2+}]_i$ decline and relaxation by 25% ($p < 0.02$; Figure 5). Changes in diastolic fluorescence were not observed, which is in agreement with previous observations in myocytes exposed to the same ISO concentration, in which diastolic $[Ca^{2+}]_i$ was measured with indo-1 (R.A. Bassani, unpublished results).

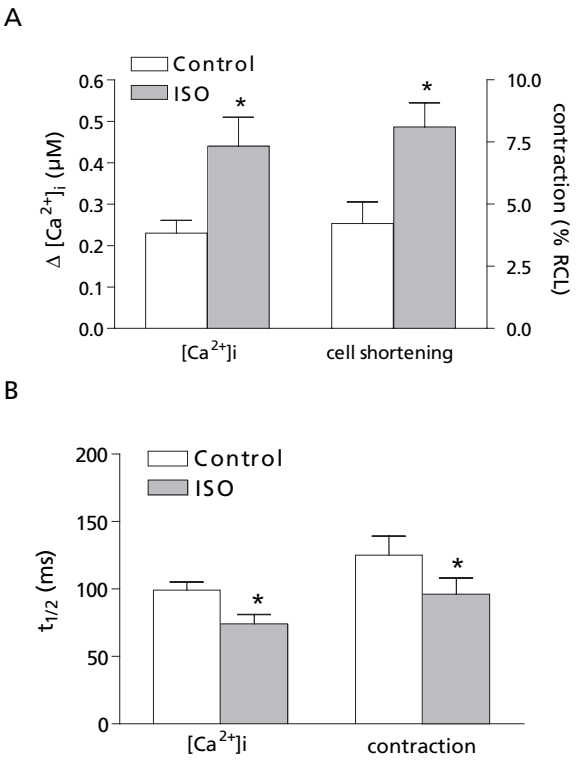

Figure 5. Positive inotropic and lusitropic effects of 10 nM isoproterenol (ISO) in rat ventricular myocytes. (A) systolic peak amplitude of $[Ca^{2+}]_i$ transients ($\Delta[Ca^{2+}]_i$) and cell shortening (% of resting cell length, RCL). (B) Half-time ($t_{½}$) values for $[Ca^{2+}]_i$ decline and relaxation. Bars are means and vertical lines represent the standard error (N = 7); *, $p < 0.05$ *vs.* control.

Discussion

The developed instrumentation showed several advantages, such as compactness, easy operation, and low cost, which was equivalent to ~10% of that of a complete system for $[Ca^{2+}]_i$ and cell shortening measurement commercially available.

An important technological improvement of the present microfluorimetry system is the use of a LED as the light source for fluo-3 excitation. LEDs may replace at a much lower cost some usually employed light sources, such as laser beams and arc lamps, while providing radiant power high enough for a good signal to noise ratio, and at a reasonably narrow wavelength band. In addition, LEDs do not generate much heat, and have high stability and a long life-span (over 11,000 hours). The technology for developing high-intensity LEDs that emit at lower wavelengths is relatively new, and was successfully incorporated in this project.

As an application of the developed instrumentation, we determined and compared the effect of β-adrenoceptor stimulation with ISO on Ca^{2+} transients and mechanical activity developed by isolated ventricular myocytes. We tested a relatively low ISO concentration (10 nM), which corresponds to ~4-fold the agonist concentration required half-maximal inotropic effect in this preparation (Carvalho *et al*., 2006) and is ~100-times lower than the concentrations usually employed for experimental β-adrenergic stimulation in the heart (Hussain and Orchard, 1997; Li *et al*., 2000; Ginsburg and Bers, 2004; Ginsburg and Bers, 2005). Thus, it is plausible to assume that 10 nM ISO would mimic a near-physiological level of cardiac sympathetic stimulation. At this concentration, ISO caused significant positive inotropic and lusitropic effects, enhancing amplitude and abbreviating the timecourse of both $[Ca^{2+}]_i$ and contractile twitch waveforms, without obvious signs of $[Ca^{2+}]_i$ overload (i.e., development of spontaneous contractions), which may interfere with membrane potential and ion channel activity, and attenuate the β-adrenergic inotropic effect (Bers, 2001).

Positive inotropic and chronotropic effects of catecholamines are the main mechanisms of cardiac output enhancement during the alarm phase of stress reaction. The positive lusitropic effect allows complete myocardial relaxation and ventricular filling under increased cardiac rate (Bers, 2001). ISO acts on sarcolemmal β-adrenergic receptors that are activated *in vivo* by the

sympathetic mediators noradrenaline and adrenaline. The signaling pathway coupled to these receptors mediates the chronotropic, inotropic, lusitropic and metabolic effects of sympathetic stimulation in the heart, via activation of the cyclic AMP-dependent protein kinase (PKA), which phosphorylates several intracellular proteins. Many of the PKA substrates are involved in cell Ca^{2+} cycling (Bers, 2001), such as: a) sarcolemmal voltage-dependent Ca^{2+} channels, in which phosphorylation increases the inward Ca^{2+} current (I_{Ca}) that acts as a trigger for SR Ca^{2+} release); b) phospholamban, an endogenous inhibitor of the SR Ca^{2+}-ATPase, which is the main transporter responsible for cytosolic Ca^{2+} removal during twitch relaxation in mammalian heart (Bassani *et al*., 1992); phosholamban phosphorylation results in increased rate of Ca^{2+} uptake by the ATPase, causing not only faster relaxation, but also increase in the SR Ca^{2+} content, and thus, in the amount of Ca^{2+} released at systole (Bassani *et al*., 1995; Ginsburg and Bers, 2004); c) troponin I: phosphorylation decreases the myofilament sensitivity to Ca^{2+}, which facilitates dissociation of the latter from the thin filament (Bers, 2001; Li *et al*., 2000). PKA-dependent phosphorylation of sarcolemmal Ca^{2+} channels and phospholamban, and consequent enhanced systolic Ca^{2+} mobilization, have been shown to be involved in β-adrenergic inotropic stimulation (Bers, 2001; Li *et al*., 2000; Luo *et al*., 1994; Sham *et al*., 1991; Viatchenko-Karpinski and Györke, 2001). On the other hand, the positive lusitropic effect seems to depend on phospholamban phosphorylation at a greater extent than on troponin I phosphorylation (Li *et al*., 2000; Luo *et al*., 1994).

In the present study, we observed that both the increase in twitch amplitude and decrease in decline $t_{1/2}$ values caused by 10 nM ISO were quantitatively similar for $[Ca^{2+}]_i$ transients and contractions. Due to the possibility of simultaneous measurement of both $[Ca^{2+}]_i$ and cell shortening, we were able to determine the shortening-$[Ca^{2+}]_i$ loop during a single twitch. The absence of major shift or distortion of this loop in the presence of ISO (albeit the loop was expanded as a consequence of the amplitude enhancement of both variables) is suggestive that ISO effects on contraction amplitude and timecourse, at the concentration used in this work, are mostly due to the changes in $[Ca^{2+}]_i$ mobilization, rather in the function of the contractile apparatus.

In summary, we present a low-cost instrument for simultaneous $[Ca^{2+}]_i$ and cell shortening measurement, which was applied in the analysis of the functional changes produced by β-adrenergic stimulation in cardiac myocytes. This system may be considered an important tool for studying excitation-contraction coupling in cardiac cells, and also for $[Ca^{2+}]_i$ measurement in other cell types.

Acknowledgements

We are grateful to Ms. Elizângela S.O. Vieira, Mr. Jean R. de Ávila and Ms. Márcia A. Queiroz (CEB/UNICAMP) for the excellent technical support. We also would like to thank Mr. Sérgio P. Moura, Eugênio C. Carrara e Mauro S. Martinazzo (CEB/UNICAMP) for developing the electrical stimulator used in this study. This work was supported by FAPESP (Proc. nº 05/52601-1) and CNPq (Proc. nº 300632/2005-3).

References

Bassani, J.W.M., Bassani, R.A., Bers, D.M. (1993), "Ca^{2+} cycling between sarcoplasmic reticulum and mitochondria in rabbit cardiac myocytes", *The Journal of Physiology*, v. 460, p. 603-621.

Bassani, J.W.M., Bassani, R.A., Bers, D.M. (1994), "Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms", *The Journal of Physiology*, v. 476, n. 2, p. 279-293.

Bassani, J.W.M., Yuan, W., Bers, D.M. (1995), "Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes", *American Journal of Physiology Cell Physiology*, v. 268, n. 5, pt. 1, p. C1313-C1319.

Bassani, R.A., Bassani, J.W.M., Bers, D.M. (1992), "Mitochondrial and sarcolemmal Ca^{2+} transport can reduce $[Ca^{2+}]_i$ during caffeine contractures in rabbit cardiac myocytes", *The Journal of Physiol*ogy, v. 453, p. 591-608.

Bassani, R.A., Bassani, J.W.M. (2002), "Contribution of Ca^{2+} transporters to relaxation in intact ventricular myocytes from developing rats", *American Journal of Physiology Heart and Circulatory Physiology*, v. 282, n. 6, p. H2406-H2413.

Berridge, M.J., Lipp, P., Bootman, M.D. (2000), "The versatility and universality of calcium signaling", *Nature Reviews. Molecular Cell Biology*, v. 1, n. 1, p. 11-21.

Bers, D.M. (2001), *Excitation-Contraction Coupling and Cardiac Contractile Force*, 2nd ed., Dordrecht: Kluwer Academic Publisher, 452 p.

Bers, D.M. (2002), "Cardiac excitation-contraction coupling", *Nature*, v. 415, n. 6868, p. 198-205.

Carvalho, B.M.R., Bassani, R.A., Franchini, K.G., Bassani, J.W.M. (2006), "Enhanced calcium mobilization in rat ventricular myocytes during the onset of pressure overload-induced hypertrophy", *American Journal of Physiology Heart and Circulatory Physiology*, v. 291, n. 4, p. H1803-H1813.

Cheng, H., Lederer, W.J., Cannell, M.B. (1993), "Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle", *Science*, v. 262, n. 5134, p. 740-744.

Fabiato, A. (1983), "Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum", *American Journal of Physiology Cell Physiology*, v. 245, n. 1, p. C1-C14.

Ginsburg, K.S., Bers, D.M. (2004), "Modulation of excitation-

contraction coupling by isoproterenol in cardiomyocytes with controlled SR Ca^{2+} load and Ca^{2+} current trigger", *The Journal of Physiology*, v. 556, pt. 2, p. 463-480.

Ginsburg, K.S., Bers, D.M. (2005), "Isoproterenol does not enhance Ca-dependent Na/Ca exchange current in intact rabbit ventricular myocytes", *Journal of Molecular and Cell Cardiology*, v. 39, n. 6, p. 972-981.

Gomes, P.A.P., Bassani, R.A., Bassani, J.W.M. (1998), "Measuring [Ca^{2+}] with fluorescent indicators: theoretical approach to the ratio method", *Cell Calcium*, v. 24, n. 1, p. 17-26.

Harkins, A.B., Kurebayashi, N., Baylor, S.M. (1993), "Resting myoplasmic free calcium in frog skeletal muscle fibers estimated with fluo-3", *Biophysical Journal*, v. 65, n. 2, p. 865-881.

Hussain, M., Orchard, C.H. (1997), "Sarcoplasmatic reticulum Ca^{2+} content, L-type Ca^{2+} current and Ca^{2+} transient in rat myocytes during β-adrenergic stimulation", *The Journal of Physiology*, v. 505, pt. 2, p. 385-402.

Kao, J.P.Y., Harootunian, A.T., Tsien, R.Y. (1989), "Photochemically generated cytosolic calcium pulses and their detection by fluo-3", *Journal of Biological Chemistry*, v. 264, n. 14, p. 8179-8184.

Li, L., Desantiago, J., Chu, G., Kranias, E.G., Bers, D.M. (2000), "Phosphorylation of phospholamban and troponin I in β-adrenergic-induced acceleration of cardiac relaxation", *American Journal Physiology Heart and Circulatory Physiology*, v. 278, n. 3, p. H769-H779.

Luo, W., Grupp, I.L., Harrer, J., Ponniah, S., Grupp, G., Duffy, J.J., Doetschman, T., Kranias, E.G. (1994), "Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of β-agonist stimulation", *Circulation Research*, v. 75, n. 3, p. 401-409.

Thomas, A.P., Delaville, F. (1991), "The use of fluorescent indicators for measurements of cytosolic-free calcium concentration in cell population and single cells", In: *Cellular Calcium – A Practical Approach*, Eds.: J.G.,McCormack, P.H., Cobbold, New York: Oxford University Press, p. 1-53.

Ricardo, R.A., Oliveira, P.X., Bassani, R.A., Bassani, J.W.M. (2006), "Compact cell image projector: application to study the relationship between stimulus interval and contractions amplitude in isolated rat cardiomyocytes", *Brazilian Journal of Biomedical Engineering*, v. 22, n.2, p. 151-160.

Sham, J.S.K., Jones, L.R., Morad, M. (1991), "Phospholamban mediates the β-adrenergic-enhanced Ca^{2+} uptake in mammalian ventricular myocytes", *American Journal Physiology Heart and Circulatory Physiology*, v. 261, p. H1344-H1349.

Shannon, T.R., Ginsburg, K.S., Bers, D.M. (2002), "Quantitative assessment of the SR Ca^{2+} leak-load relationship", *Circulation Research*, v. 91, n. 7, p. 594-600.

Song, L., Hennink, E.J., Young, I.T., Tanke, H.J. (1995), "Photobleaching kinetics of fluorescein in quantitative fluorescence microscopy", *Biophysical Journal*, v. 68, n. 6, p. 2588-2600.

Takahashi, A., Camacho, P., Lechleiter, J.D., Herman, B. (1999), "Measurement of intracellular calcium", *Physiological Reviews*, v. 79, n. 4, p. 1089-1125.

Viatchenko-Karpinski, S., Györke, S. (2001), "Modulation of the Ca^{2+} -induced Ca^{2+} release cascade by β-adrenergic stimulation in rat ventricular myocytes", *The Journal of Physiology*, v. 533, pt. 3, p. 837-848.